Proc. Natl. Acad. Sci. USA
Vol. 73, No. 10, pp. 3584–3588, October 1976
Cell Biology

The relationship between *in vitro* cellular aging and *in vivo* human age

(*in vitro* lifespans/replicative capabilities/old and young donor human skin fibroblasts/early and late passage WI-38 cells)

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Communicated by James D. Ebert, July 12, 1976

ABSTRACT **Differences between early and late passage cell cultures on the organelle and macromolecular levels have been attributed to cellular "aging." However, concern has been expressed over whether changes in diploid cell populations after serial passage *in vitro* accurately reflect human cellular aging *in vivo*. Studies were therefore undertaken to determine if significant differences would be observed in the *in vitro* lifespans of skin fibroblast cultures from old and young normal, nonhospitalized volunteers and to examine if parameters that change with *in vitro* "aging" are altered as a function of age *in vivo*. Statistically significant ($P \ll 0.05$) decreases were found in the rate of fibroblast migration, onset of cell culture senescence, *in vitro* lifespan, cell population replication rate, and cell number at confluency of fibroblast cultures derived from the old donor group when compared to parallel cultures from young donors. No significant differences were observed in modal cell volumes and cellular macromolecular contents. The differences observed in cell cultures from old and young donors were quantitatively and qualitatively distinct from those cellular alterations observed in early and late passage WI-38 cells (*in vitro* "aging"). Therefore, although early and late passage cultures of human diploid cells may provide an important cell system for examining loss of replicative potential, fibroblast cultures derived from old and young human donors may be a more appropriate model system for studying human cellular aging.**

The limited lifespan of cultured human diploid fibroblasts *in vitro* has led to their extensive utilization as a model system for studying human cellular aging (1–3). These human fetal lung fibroblasts in their last passages are often referred to as representing "aged" cell populations (4–6). Differences between early and late passage cell cultures on the organelle and macromolecular levels have been attributed to cellular "aging" (4–6). However, concern has been expressed over whether changes in diploid cell populations after serial passage *in vitro* accurately reflects human cellular aging *in vivo* (7–9). One of the most frequently cited justifications for the use of cultured fibroblasts for studying human cellular aging is the reported decline in the cumulative number of cell population replications *in vitro* and earlier onset of cell culture senescence with the increasing age of the cell culture donor (10, 11). Recently, however, the statistical analysis and population selection used in these studies have been questioned (7, 8).

The studies to be described in this report had two major goals: (*i*) to determine if statistically significant differences would be observed in the onset of cell culture senescence and the cumulative replication capacity of fibroblast cultures derived from old and young normal, nonhospitalized human volunteers; and (*ii*) to examine if parameters that change with increased *in vitro* "aging" are altered as a function of *in vivo* age. This latter aspect was approached by comparing cell population replication rate, percent replicating cells, cell number at confluency, cell volume, and cellular macromolecular contents in cell cultures from old and young human donors (aging *in vivo*) as well as in early and late passage cell cultures ("aging" *in vitro*).

Abbreviation: CPD, cell population doublings.

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MATERIALS AND METHODS

Cell Culture. After informed consent had been obtained, skin punch biopsies, 2 mm in diameter, were performed on the inner aspect of the upper arm of nonhospitalized male volunteers. The subjects were interviewed prior to biopsy, and those individuals with a history of diabetes or who were receiving steroid medication were not included in this study. The skin specimen was divided into four equal explants and two each were placed between coverglass sandwiches in Leighton tubes. Fibroblast outgrowth from the explanted skin was measured at weekly intervals with a micrometric ocular attachment to a Zeiss inverted microscope. Explant outgrowth was defined as furthest migration of cells in micrometer units per time period (1 unit = 0.317 mm). When fibroblast outgrowth formed a confluent monolayer, the cells were harvested and transferred to 25 cm^2 plastic flasks (Falcon, 3012). Cell cultures were subcultured in 25 cm^2 flasks at a 1:4 split ratio with the initial confluent monolayer designated as one cell population doubling (1 CPD). All cultures were coded by number, and the code was broken upon completion of data collection.

Cultures of human fetal lung fibroblasts (WI-38) at early (19 CPD) and late (39 CPD) passages were obtained from Dr. L. Hayflick and subcultured at 1:4 split ratio prior to analyses. Upon further subcultivation, these cultures had remaining lifespans of approximately 31 and 11 CPD, respectively.

Cells were cultured in Eagle's minimal essential medium, supplemented with nonessential amino acids, glutamine, 50 μg/ml of Aureomycin (GIBCO), and 10% fetal bovine serum (Flow Labs) in a 5% CO_2:95% air environment. Two batches of fetal bovine serum with equal growth-promoting capabilities (tested by examining cell growth kinetics on parallel cultures) were used for the entire study. Subcultivation involved washing of the monolayer with calcium and magnesium-free phosphate-buffered saline, pH 7.4, addition of sufficient 0.1% pronase to just cover the monolayer, and neutralization of proteolytic activity with complete medium after cell detachment. Cell cultures were tested for and found free of mycoplasma contamination by the uridine/uracil technique (12).

During the initial passages, cell cultures were subcultured weekly when the monolayer reached confluency. If cultures failed to reach confluency in 1 week, the culture was termed "senescent," fresh medium was added weekly, and the cumulative CPD recorded. After 4 weeks, if the cell culture had not reached confluency, the cell strain was considered to have

Table 1. Characteristics of skin explants and cell cultures derived from young and old human donors

	Young donors (21–36 yr)	Old donors (63–92 yr)
Successful cell outgrowth from explants	23/34	24/39
Cell cultures that senesced before 10 CPD	0/23	3/24
Explant outgrowth at 1 week (units)	2.44 ± 0.30 (26)*†	1.44 ± 0.15 (29)†
Onset of senescent phase (*A*) (CPD)	35.2 ± 2.1 (23)	20.0 ± 2.0 (24) 22.5 ± 1.7 (21)‡
Onset of senescent phase (*B*) (CPD)	41.6 ± 2.4 (23)	26.3 ± 2.6 (24) 29.6 ± 2.1 (21)‡
In vitro lifespan (CPD)	44.6 ± 2.5 (23)	29.8 ± 2.9 (24) 33.6 ± 2.2 (21)‡
In vitro lifespan (days)	273 ± 11 (23)	218 ± 14 (24) 236 ± 12 (21)‡

* Values are expressed as mean ± standard error of the mean. Numbers within parentheses indicate the number of individual cell cultures examined.

† Includes several measurements of outgrowth from two explants taken from the same donor.

‡ If only cultures with >10 CPD included.

"senesced" and the lifespan *in vitro* in cumulative CPD was recorded.

Cellular Measurements. Cell population replication rate, percent replicating cells, cell number at confluency, cell volumes, and cellular macromolecular contents were determined on old and young donor skin fibroblast cultures during their initial 10 CPD. Cultures that senesced before 10 CPD were excluded from these studies.

To determine cell population replication rate and cell number at confluency, we inoculated 5×10^4 fibroblasts into 9 cm^2 plastic petri dishes (Falcon). Fresh medium was added every third day to insure sustained rapid growth. Two replicate dishes were harvested each day, and the cell number per dish was measured with a model ZBI Coulter counter.

The percent replicating cells was determined by described autoradiographic techniques (13). In brief, tritiated thymidine

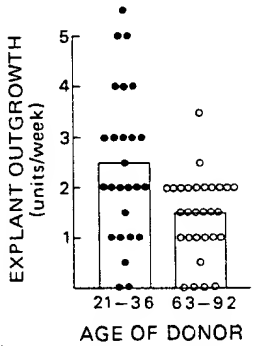

FIG. 1. Migration rates of fibroblasts from skin explants derived from young and old human donors. The bars in Figs. 1, 2, 3, 6, and 7 represent the mean values, while solid circles are individual values from young donor cultures and open circles represent values from old donor cultures.

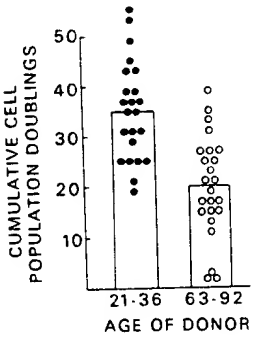

FIG. 2. Onset of cell culture senescence (in cumulative cell population doublings) defined as failure to reach confluency 1 week after subcultivation.

(0.05 μCi/ml, specific radioactivity 2 Ci/mmol, Schwarz Biochemical) was added in medium to cells on coverglasses for 24–72 hr, during the logarithmic phase of cell culture growth. Individual coverglasses were removed at 24, 48, and 72 hr, then fixed with methanol:acetic acid (3:1), washed with 4% perchloric acid, dipped in NTB-2 emulsion (Kodak), exposed for 5 days, then developed, and stained with Giemsa. The percent of the total cell population that had labeled nuclei (covered with more grains than background) was calculated.

Cell volume distributions were determined at confluency, after the cells were detached from their monolayers with Pronase (0.1%), with a Coulter counter and channelizer using fixed L cells (Cell-chex) and mulberry pollen as standards (14).

Cellular macromolecular contents were measured as described by the Lowry (protein) diphenylamine (DNA) and orcinol (RNA) techniques (15).

RESULTS

Cell outgrowth

There was a slight, but not statistically significant, decrease in the frequency of successful fibroblast outgrowth from the skin biopsies obtained from the older donor group (ages 63–92) when compared with the younger donor group (ages 21–36, Table 1). However, migration of fibroblasts from the explant was considerably more rapid in the younger donor group. Although overlap exists between values for migration rate between groups (Fig. 1), this decreased migration rate of cell cultures derived from old donors was statistically significant ($P < 0.005$).

After transfer of the cells from Leighton tubes to tissue culture flasks, all the cultures derived from young donors replicated rapidly during their initial 20 CPD. In contrast to this uniform, early rapid replication of cell cultures from young donors, three of the 24 cultures derived from the old donor group senesced well before 10 CPD (Table 1).

Onset of cell culture senescence

Cell culture senescence was defined as the number of CPD at which the cell culture failed to reach confluency at either (*A*) 1 week after subcultivation or (*B*) 2 weeks after subcultivation (Table 1). By either definition, cell cultures from older donors reached the senescent phase of their *in vitro* lifespans at significantly lower levels of cumulative CPD. Even if those cell cultures derived from old donors that senesced before 10 CPD are omitted from analysis, this statistically significant earlier onset of cell culture senescence remains ($P < 0.001$). As in the cell migration studies, overlap exists between old and young donor cultures in the onset of cell culture senescence (Fig. 2).

Table 2. Determinations of *in vitro* "aging" indices on skin fibroblast cultures derived from young and old human donors and WI-38 cell cultures at early and late *in vitro* passage*

	Skin fibroblast cultures		WI-38 cell cultures	
Level of *in vitro* passage (CPD)	5–10	5–10	20–29	40–49
Age of fibroblast donor (yr)	28.1 ± 1.0 (18)	78.9 ± 1.7 (18)	0†	0†
Percent replicating cells‡	87.7 ± 1.6 (7)	79.6 ± 2.5 (7)	97	62
Cell population doubling time (hr)	20.8 ± 0.8 (18)	24.3 ± 0.9 (18)	17.1 ± 0.8 (6)	25.6 ± 2.8 (5)
Cell number at confluency (10^4 cells/cm^2)	7.31 ± 0.42 (18)	5.06 ± 0.52 (17)	18.01 ± 1.33 (5)	8.64 ± 1.47 (5)
Modal cell volume (μm^3)	2935 ± 88 (18)	3131 ± 109 (17)	1930 ± 20 (5)	2655 ± 234 (4)
Cellular RNA content (pg/cell)	28.7 ± 1.9 (16)	29.3 ± 1.7 (13)	21.5 ± 1.6 (29)	45.6 ± 3.3 (25)
Cellular protein content (pg/cell)	573 ± 33 (16)	576 ± 38 (13)	490 ± 26 (29)	883 ± 55 (25)

* Values, except for level of *in vitro* passage and percent replicating cells (in the case of the WI-38 cells), are expressed as the mean ± standard error of the mean. The numbers within parentheses indicate the number of individual cell cultures examined.
† These fetal lung cultures were obtained from an abortus at approximately three (3) gestational months.
‡ Labeled nuclei after 24 hr in [^{3}H]thymidine.

Total replicative capacity (*in vitro* lifespan)

The *in vitro* lifespans of the cell cultures derived from both old and young donors, expressed as cumulative cell population doublings, are displayed in Fig. 3. The mean *in vitro* lifespan of the fibroblast cultures derived from the young donor group was 50% greater than that observed in the old donor group. If those cultures that grew poorly and senesced before 10 CPD are eliminated from analysis, a statistically significant difference ($P < 0.05$) (expressed as CPD or as days in culture) is still present between cultures derived from old and young donors (Table 1).

Percent replicating cells ([^{3}H]thymidine-labeled nuclei)

Since autoradiographic determination of the percent replicating cells in a culture varies as a function of [^{3}H]thymidine incubation time, analyses were performed on all cell cultures at three separate incubation periods. In Fig. 4A, the percent [^{3}H]thymidine-labeled nuclei, reflecting percent replicating cells, of cultures derived from young and old human donors is seen. For a comparison with *in vitro* cellular "aging" the results of determinations performed on early and late passage WI-38 cell cultures are presented in Fig. 4B. Measurements at 24 hr are probably the most informative since cell population doubling times of both skin fibroblast cultures (old and young donors) and WI-38 cells ranged from 17 to 31 hr. Although a significant increase in the percent replicating cells is observed after a 24-hr incubation period in cell cultures derived from young donors ($P < 0.05$), it does not approach the magnitude of the difference between early and late passage cell cultures (Table 2).

Cell population replication rate, cell number at confluency, and cell volume

Typical growth curves of cell cultures derived from a young and an old donor are seen in Fig. 5. As in most human diploid fibroblast cultures, there is a drop in cell number after transfer, followed by a phase of rapid growth and, finally, a plateauing as the culture reaches confluency. The drop in cell number after transfer, sometimes referred to as the "plating efficiency," was similar in both young and old donor cell cultures. In Fig. 6, the distribution of the cell population replication rates of the cell cultures derived from old and young donors determined during the rapid growth phase is seen. Although not as striking as the differences observed between early and late passage WI-38 cell cultures, the mean cell population doubling time for old donor cultures was significantly increased ($P < 0.01$) when compared to the value obtained from young donor cultures (Table 2). Even more impressive than the difference in cell population replication rate was the marked differences in cell number at confluency that was observed between old and young donor cell cultures (Fig. 7). While not as spectacular as the difference between early and late passage WI-38 cells, the mean cell number at confluency in young donor cell cultures was almost 50% higher and statistically significant at $P < 0.005$ (Table 2).

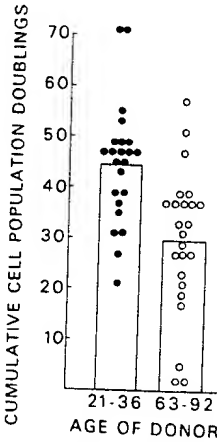

FIG. 3. *In vitro* lifespans of skin fibroblast cultures in cumulative number of cell population doublings.

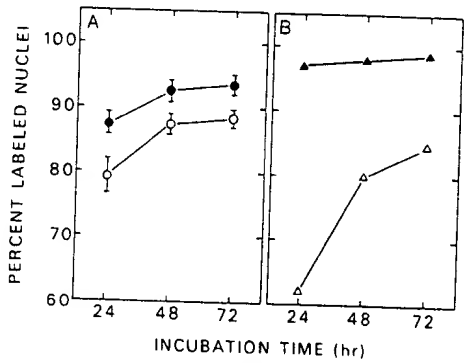

FIG. 4. Percent labeled nuclei after varying incubation periods with [^{3}H]thymidine of (A) skin fibroblast cultures derived from young (●) and old (○) human donors and (B) early (19 CPD, ▲) and late (45 CPD, △) passage WI-38 cells. Values in (A) represent the mean and standard error of the mean of seven individual cultures.

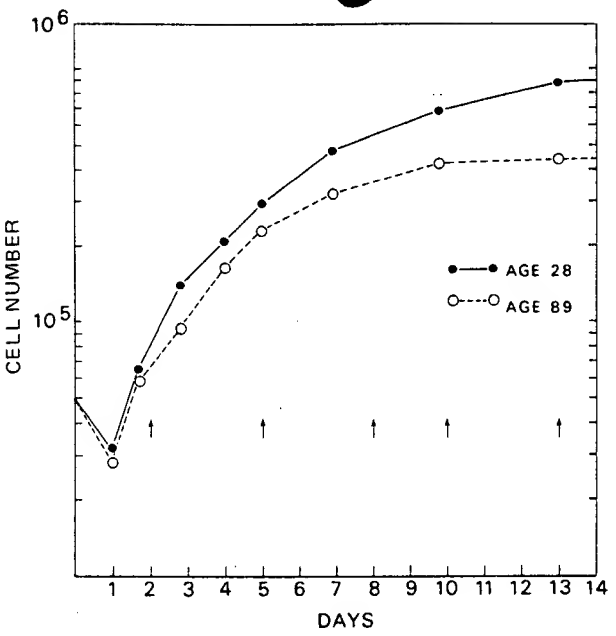

FIG. 5. Cell population growth curves of skin fibroblast cultures derived from a young and an old donor. Arrows indicate change of medium.

Although modal cell volumes were larger in old donor cultures, the difference was not statistically significant, $P > 0.05$ (Table 2). As has been previously reported (14), modal cell volumes of late passage WI-38 cells were significantly larger than those of early passage cells.

Macromolecular contents

In contrast to the marked increase in cellular RNA and protein contents observed in late passage WI-38 cell cultures (15), the contents of these macromolecules in cultures derived from old and young human donors were essentially identical. DNA contents, which did not change as a function of *in vitro* "aging" were also not significantly affected by donor age (11.2 pg per cell in young donor cultures in contrast with 10.6 pg per cell in old donor cultures).

DISCUSSION

In the above studies, considerable variation is observed with overlap between young and old donor groups present in nearly every parameter measured. This is not surprising since studies of various physiologic parameters as a function of normal human aging have similarly demonstrated overlap between young and old groups (16, 17). This may be related to the use

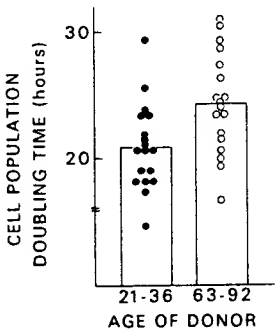

FIG. 6. Cell population doubling times of cell cultures derived from young and old donors.

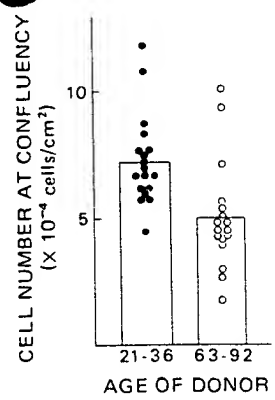

FIG. 7. Cell number at confluency of skin fibroblast cultures derived from young and old donors.

of chronologic age as an indicator of aging. Clinicians and physiologists have long been aware that the biologic age of an individual may be quite disparate from his chronologic age (18). In fact, several longitudinal and cross-sectional studies of aging have addressed themselves to obtaining an index of biologic age (19–21). Another probable source of the observed variation is the genetic heterogeneity of human subjects.

Despite this variation, our studies comparing cell cultures derived from young and old human donors clearly indicate that statistically significant differences can be observed *in vitro* as a function of donor age *in vivo*. Among the parameters that showed the greatest correlation with donor age was the rate of migration of fibroblasts from the explanted tissue. This age-related decline in fibroblast migration has also been documented in other species (22). One potential criticism of these findings is the known decrease in cellularity that occurs in a variety of tissues with normal aging (23, 24). However, since we measured migration distance and not the number of cells that migrated from the explant, we feel that our results reflect a real difference in migration ability and not merely a secondary effect of decreased cellularity. A 2-fold difference in explant cellularity, if present, could lead to a culture from an older individual having to undergo one additional cell population doubling to reach confluency before the initial passage. However, it is unlikely that this difference in one additional cell population doubling would significantly alter the results of our studies of other "aging" parameters *in vitro*.

By two separate criteria, a significantly earlier onset of cell culture senescence was observed in cultures derived from the older donor group. Similarly, measurements of total replicative capacity revealed an age-related decline in the cumulative number of cell population doublings. Since skin is an actively dividing tissue, this may reflect more previous CPD *in vivo* for skin fibroblasts from old donors and, therefore, when subsequently placed into culture *in vitro* fewer remaining CPD. Similarly, the prolonged cell population doubling rate and decreased percent replicating cells of early passage cell cultures derived from old donors may be related to their increased number of previous replications *in vivo* since cell population replication declines with increasing CPD (13).

The diminished cell number at confluency observed in cultures derived from old donors is an interesting finding. As in late passage cell cultures, this parameter is the most consistently altered. Although this decline in cell number at confluency is probably related to the increasing cell volumes observed in both old donor skin fibroblast and late passage WI-38 cell cultures, one should not exclude the possibility that these cultures have an increased sensitivity to contact inhibition.

The fact that statistically significant differences were obtained as a function of donor age in explant outgrowth, onset of cell culture senescence, *in vitro*, lifespan cell population doubling time, percent replicating cells, and cell number at confluency may well be related to the standardization of the conditions for explantation and subcultivation of the skin fibroblast cultures. The importance of obtaining cell cultures from nonhospitalized, relatively healthy donors should also be reemphasized, since it has been demonstrated that disease states, such as diabetes, can alter *in vitro* lifespan as well as other *in vitro* parameters (10).

At this point, it is important to note that our *in vitro* studies of cell cultures as a function of age *in vivo* do not reveal the magnitude or extent of differences that are observed in comparative studies performed on early and late passage human fetal lung fibroblasts (Table 2). Differences in percent replicating cells, cell population doubling time, and cell number at confluency, although statistically significant, do not approach the differences that are observed as a function of *in vitro* "aging." Measurements of cell volumes revealed only minimal differences as a function of donor age in contrast to the significant increase that is observed during the lifespan of WI-38 cells. Determinations of macromolecular contents did not reveal any difference between cell cultures derived from young and old human donors. By contrast, *in vitro* "aging" produced approximately 2-fold increases in both cellular protein and RNA contents.

Some of these differences between "*in vivo*" and "*in vitro*" aging systems may be due to differences between fibroblast cultures derived from different organs (E. L. Schneider, unpublished work) since WI-38 was established from fetal lung tissue. However, this cell strain was selected for comparison because of its wide use in studying *in vitro* cellular "aging."

In conclusion, skin fibroblasts derived from old human subjects showed statistically significant alterations in tissue culture when compared with cells derived from young donors. This supports the continued use of fibroblast cultures to study human cellular aging. However, it is important to note that these alterations are quantitatively and qualitatively distinct from the differences observed in cultured human fetal lung fibroblasts as a function of *in vitro* "aging." In addition, there were several parameters that were altered as a function of *in vitro* "aging" that were not altered *in vitro* as a function of donor age *in vivo*. Therefore, although early and late passage human diploid cultures may provide an important cell system for examining loss of replicative potential, fibroblast cultures derived from old and young human donors may be a more appropriate model system for studying human cellular aging.

Most of the volunteers whose cell cultures were used in these studies are subjects in an ongoing longitudinal aging program. It will therefore be of considerable interest to examine how the various indices *in vitro* correlate with physiologic studies performed on the same individuals. Although it may be many years before sufficient information is obtained, it will also be important to analyze if parameters, such as *in vitro* lifespan will have any relationship to *in vivo* lifespan or to the development of age-related disorders.

We thank Drs. Reubin Andres, Nathan Shock, and Arthur Norris and the members of the Baltimore Longitudinal Study for their cooperation in allowing us to obtain skin biopsies on a relatively healthy population; Mses. Karen Braunschweiger, Lynn Shiller, and Judith Kusterer and Messrs. Kin-sing Au, Kevin Johnson, and John Chaillet for technical assistance; and Dr. L. Hayflick, whose NIA contract supplied WI-38 cells at different levels of *in vitro* passage.

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